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Utilization of Ionic Liquids for Pathogen Neutralization

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I. INTRODUCTION

Summary

Biofilm-protected microbial infections in skin are a serious health risk that remains to be adequately addressed. The lack of progress in developing effective treatment strategies is largely due to the transport barriers posed by the stratum corneum of the skin and the biofilm. In this work, we report on the use of ionic liquids (ILs) or deep eutectic solvents (DESs) for biofilm disruption and enhanced antibiotic delivery across skin layers. We report the on the syntheses of ILs, analysis of relevant physicochemical properties, and the neutralization effect on two Gram-negative biofilm-forming pathogens: *Pseudomonas aeruginosa* and *Salmonella enterica* serovar typhimurium LT2. The ILs were also examined for cytotoxicity, skin irritation, delivery of antibiotics through the skin and treatment of biofilms in a wound model. In summary, our team identified one DES, a 2:1 ratio of geranate:choline (IL**21**, Figure 1), that exhibited anti-biofilm properties, were relatively non-toxic to human cell lines *and* was able to deliver an antibiotic, cefadroxil, through multiple skin layers. This work establishes a novel paradigm of use of IL/DES for topical drug delivery and potential for pathogen neutralization for difficult to treat infections.

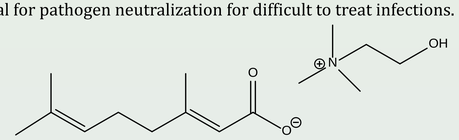

Figure **1**. Structures of geranate and choline. A 2:1 ratio provides IL**21** (deep eutectic solvent)

Background

Ionic liquids are increasingly recognized as suitable materials for multiple biological applications [1]. Based upon the tunable nature of the ILs, by varying both the anionic and cationic component, the formulations result in differing physicochemical properties of the material. Biofilms formed from bacteria are composed of a structurally complex extracellular polysaccharide matrix as a protection mechanism for the microbe. Biofilm-protected microorganisms are thought to be responsible for up to 65% of all bacterial infections in humans and are typically 50-500 times more resistant to antimicrobials than unprotected (planktonic) bacteria [2,3]. We hypothesize ionic liquids (ILs) will effectively disrupt the biofilm leading to more efficient antibiotic delivery and, hence, increased levels of cell death.

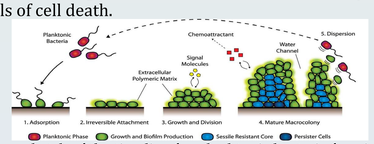

Figure **2**. Cartoon sketch of the timeline for planktonic bacteria forming biofilms.

Objectives

Design and synthesis of ILs/DESs

- Selection of anion and cation components
- Characterization of formulations

Pathogen neutralization studies

- Application of ILs/DESs to 24 and 72-hour biofilms (Gram negative)
- Microscopy
- Irritation/cytotoxicity effects

Transdermal drug delivery studies

- Assessment of skin irritation using EpiDerm®
- Biofilm growth on skin and treatment

II. APPLICATIONS OF IONIC LIQUIDS

Ionic Liquid Formulations

Table 1: Deep eutectics and ionic liquids synthesized and tested for biological activity against *P. aeruginosa* and *S. enterica* biofilms.

#	Component 1	Component 2	Material Name	Advantages
1			1-butyl-1-methylpyrrolidinium bistrifilimide (BMP-NTf_2)	low viscosity; prior use with proteins
2			Benzethonium Chloride-$(ZnCl_2)_2$ (BZBN)	components with antibacterial activity; both components are FDA-approved
5			Choline Malonate	low human toxicity; low viscosity
6			Choline Urea	low human toxicity; disruption of hydrogen bonds
7			1-hexyl-3-methylimidazolium Chloride (HMIM-Cl)	low viscosity; signs of hydrogen-bond disprution
12			Choline Bistriflimide (Choline-NTf_2)	low viscosity; cation has low toxicity
13			Choline Hexanoate	low viscosity
14			Choline Oleate	skin penetration; low human toxicity
19	$[P(C_{14}H_{29})(C_6H_{13})_3]^+$		Tetraalkylphosphonium Oleate	low viscosity, skin penetration, new material
20	$[P(C_{14}H_{29})(C_6H_{13})_3]^+$		Tetraalkylphosphonium Hexanoate	low viscosity, skin penetration, new material
21			Choline Geranate	new material, terpene derivative
22	$[P(C_{14}H_{29})(C_6H_{13})_3]^+$		Tetraalkylphosphonium Geranate	low viscosity, new material

Table 2. Physicochemical properties of ILs/DESs used for these studies

IL	viscosity (cP)	density (g/mL)	conductivity (mS/cm)	ionic strength (M)	molecular weight
1	72.72	1.39	1.99	3.28	422.4
2	8176	1.4	0.026	2.44	1439
5	920.1	1.27	0.429	5.2	243.7
6	1386	1.21	0.580	2.32	259.7
7	679.5	1.01	0.34	4.96	202.7
12	125	1.54	1.46	4.4	348.9
13	180.9	1.01	0.816	3.02	334.5
14	162.3	0.98	0.0871	1.47	667.1
19	300	0.882	0.0162	1.24	765.27
20	154	0.912	0.488	1.63	559.02
21	1345	0.99	0.0431	3.39	438.63
22	122.3	0.931	0.156	1.43	651.09
mineral oil	35	0.8	0	0	400

The anionic and cationic components of each IL/DES were chosen based upon the goal of producing fluid materials that would have both anti-biofilm activity and the ability to facilitate transdermal drug delivery.

Anti-biofilm Assays

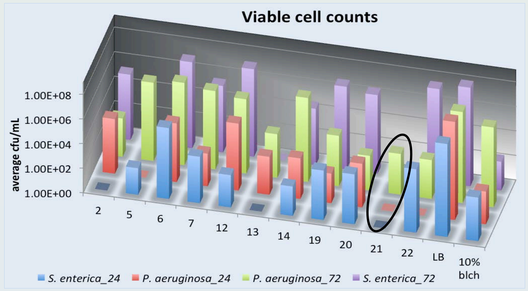

Figure **3**. Viable cell counts following 2 hour IL/DES challenge, sonication and recovery. Average cfu/mL cell counts for n=6, all data points. Error = std dev of n=6.

The Calgary method was employed for biofilm growth—*P. aeruginosa* and *S. enterica* were grown for either 24 or 72 hours in MBEC plates using published procedures.

General Method: 200 µL of each IL/DES in our panel was transferred to each well of the 96-well plate and the PEG lid harboring the respective biofilm carefully replaced and sealed with a gas permeable membrane. Following a two hour incubation at 37 ºC with shaking at 225 rpm, the biofilm released from the PEG lid into a fresh 96-well plate by sonication with a Misonix® 3000 fitted with a microplate horn. Cell viability was assessed by enumeration on LB agar plates.

General Trend: The biofilms grown for 24 hours were more susceptible to neutralization than the 72-hour biofilms when exposed to neat IL/DESs over the two hour period. The DESs **21, 13** and **5** proved to have both remarkable antimicrobial activity (Figure 3) and minimal cytotoxicity effects to human cell lines (Figure 5).

SEM images of IL-treated *P. aeruginosa*

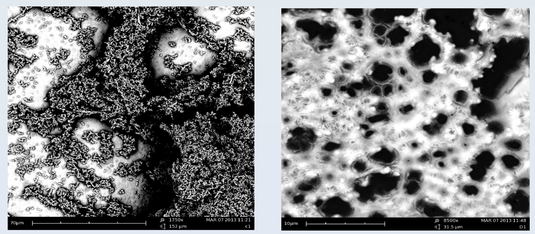

Figure 4. **Vacuum SEM images of IL-treated, 120 hour biofilms.** Biofilms were grown using slight modification of published procedures [5,6]. Images courtesy of Rico Del Sesto, Dixie State University.

72 hour *S. enterica* and *P. aeruginosa* biofilm

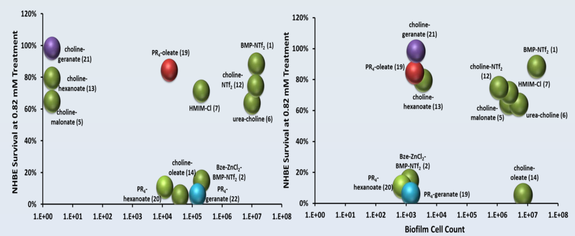

Figure 5. Correlation of IL/DES cytotoxicity effects on normal human bronchial epithelial (NHBE) cells and anti-biofilm behavior. IL/DESs were diluted to 0.82 mM (all ILs are soluble in media at this concentration) for the cytotoxicity studies. Cell cytotoxicity was measured using both the LDH activity assay and WST-1 mitochondrial assay.

III. TRANSDERMAL DRUG DELIVERY

Ionic Liquid/Deep Eutectic Solvent

Ionic liquids/deep eutectics were assayed for their ability to deliver antibiotics through multiple skin layers. Further, cytotoxicity effects of the ILs/DESs on normal human bronchial epithelial (NHBE) cells and skin irritation potential on human epidermal cultures (EpiDerm®) revealed IL**21** to have optimal antibacterial, cytotoxicity and skin permeation properties.

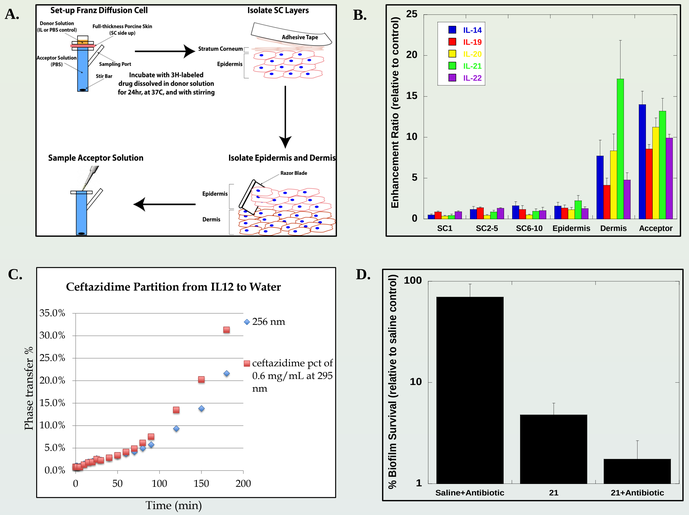

Figure 6**A.** Depiction of IL application to skin and transdermal drug delivery. **B**: Tritium-labeled cefadroxil penetration into varying skin layers. **C.** Phase transfer of ceftazidime from IL12 to the aqueous environment. Transfer kinetics monitored by UV spectroscopy. **D.** *P. aeruginosa* biofilm treatment with IL21 when grown on a wounded MatTek Epiderm FT™ human skin equivalent tissue.

Conclusions & Future Directions

- Brief 2 hour IL/DES exposures to the 24- and 72-hour biofilms resulted in significant reductions in viable cell counts.
- Weak correlations between IL conductivity and anti-biofilm properties.
- IL-21 shows promise for both pathogen neutralization within biofilms and transdermal drug delivery—with minimal cytotoxicity effects.
- Moving to Gram-positive biofilm-forming pathogens; e.g. *Staphylococcus aureus*
- IL-antibiotic stability studies are in progress.

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